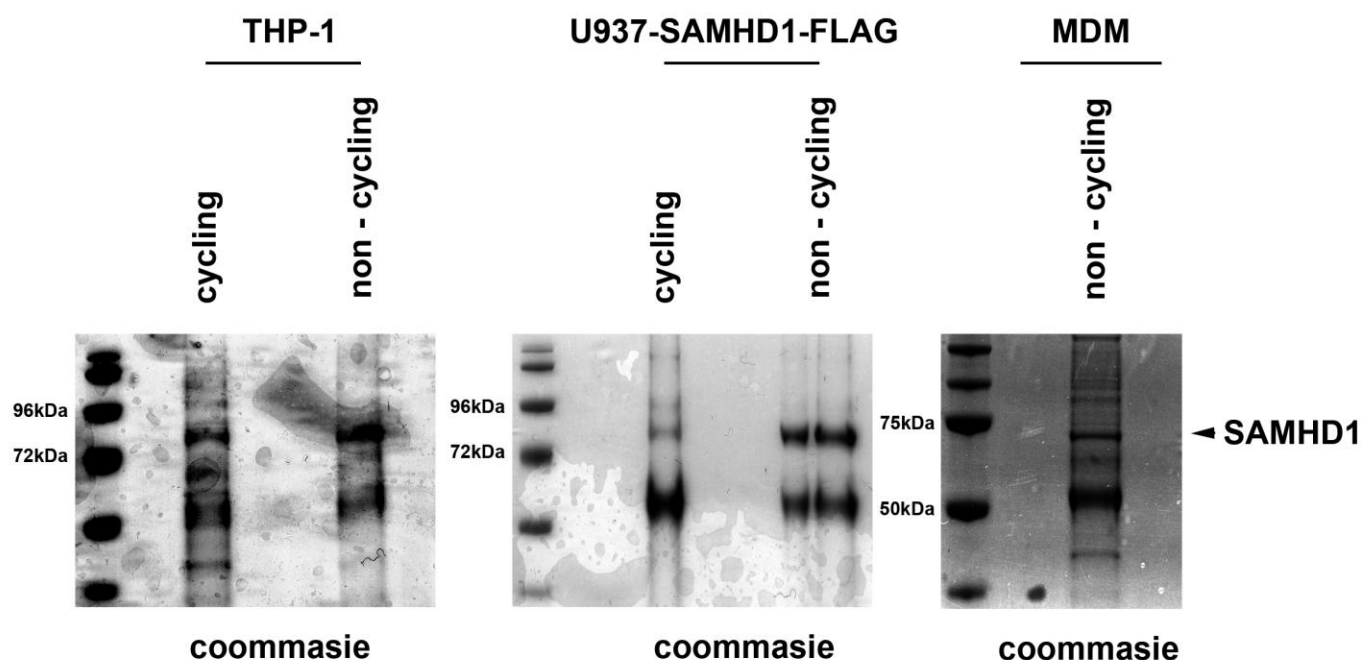


Cell Host and Microbe, Volume 13

## **Supplemental Information**

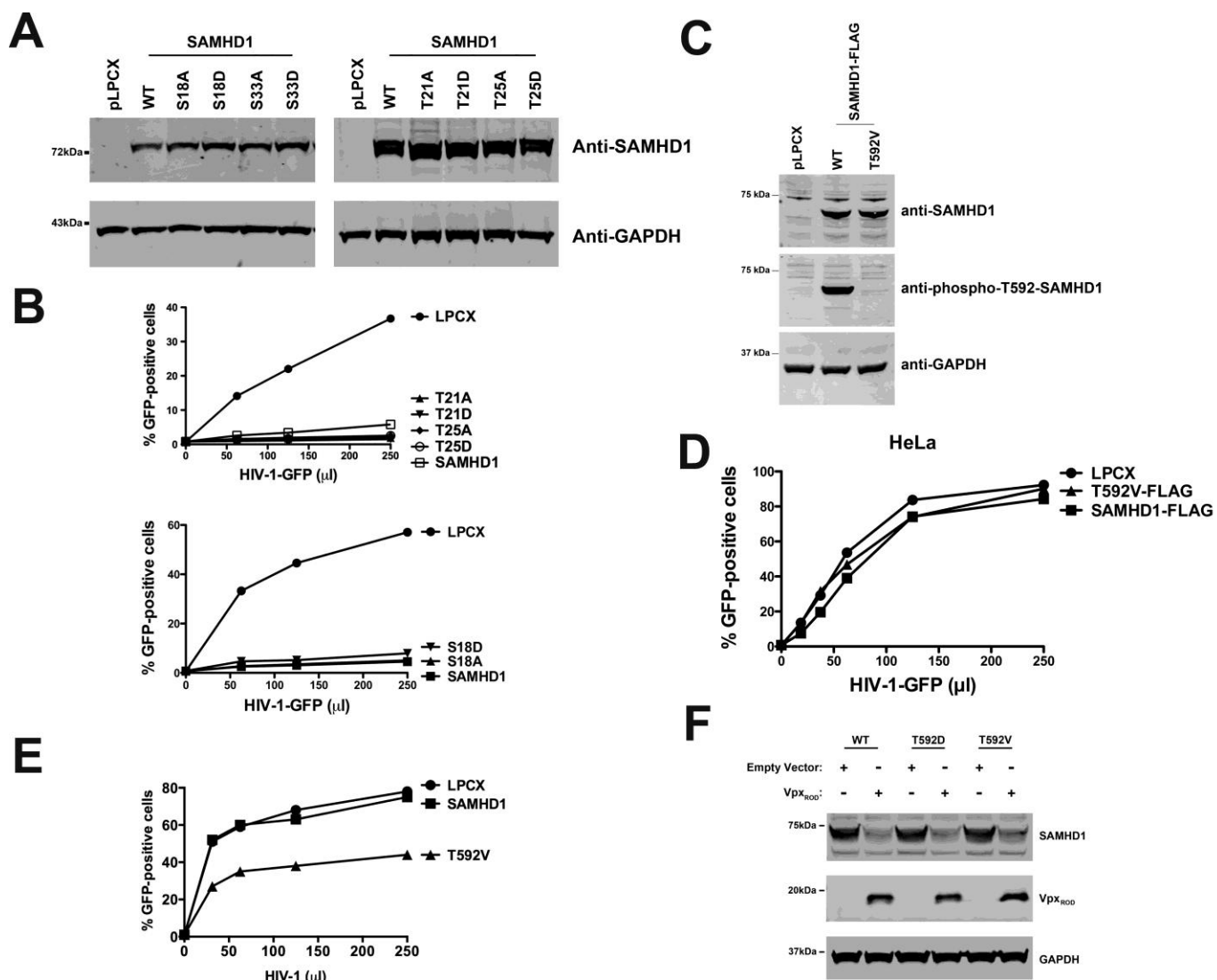
### **The Retroviral Restriction Ability of SAMHD1, but Not Its Deoxynucleotide Triphosphohydrolase Activity, Is Regulated by Phosphorylation**

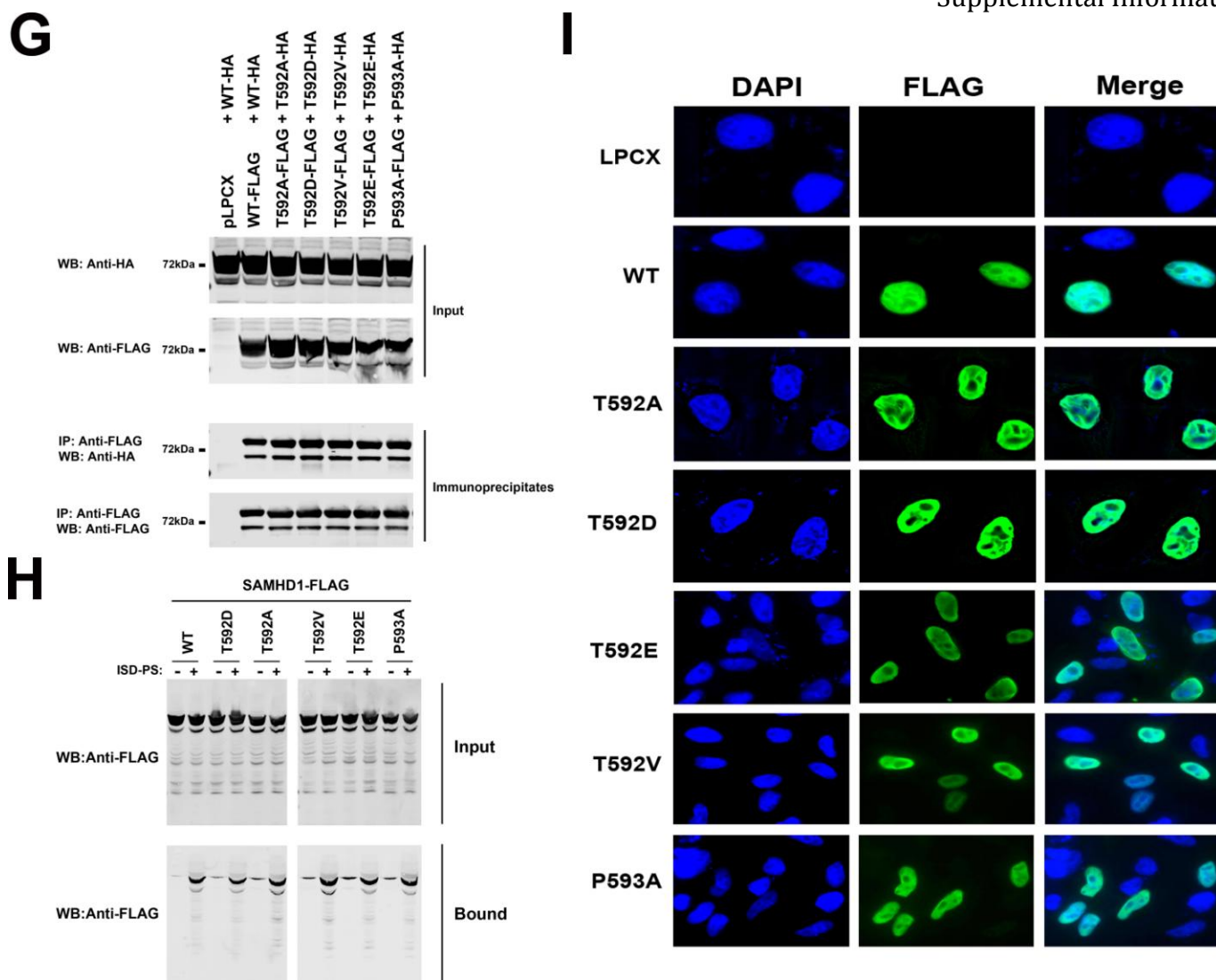
Tommy E. White, Alberto Brandariz-Núñez, Jose Carlos Valle-Casuso, Sarah Amie, Laura Anh  
Nguyen, Baek Kim, Marina Tuzova, and Felipe Diaz-Griffero



**Figure S1. Phosphorylation State of Endogenously and Exogenously Expressed SAMHD1, Related to Figure 1**

THP-1 cells, U937 cells stably expressing SAMHD1-FLAG, and monocyte-derived macrophages were used to immunoprecipitate SAMHD1. Immunoprecipitations were performed using anti-FLAG or anti-SAMHD1 antibodies when appropriate. Immunoprecipitates were resolved by SDS-PAGE gels and proteins were stained using the SimplyBlue™ SafeStain. Bands corresponding to the molecular weight of SAMHD1 were excised and digested for mapping of phosphorylation sites by mass spectrometry.

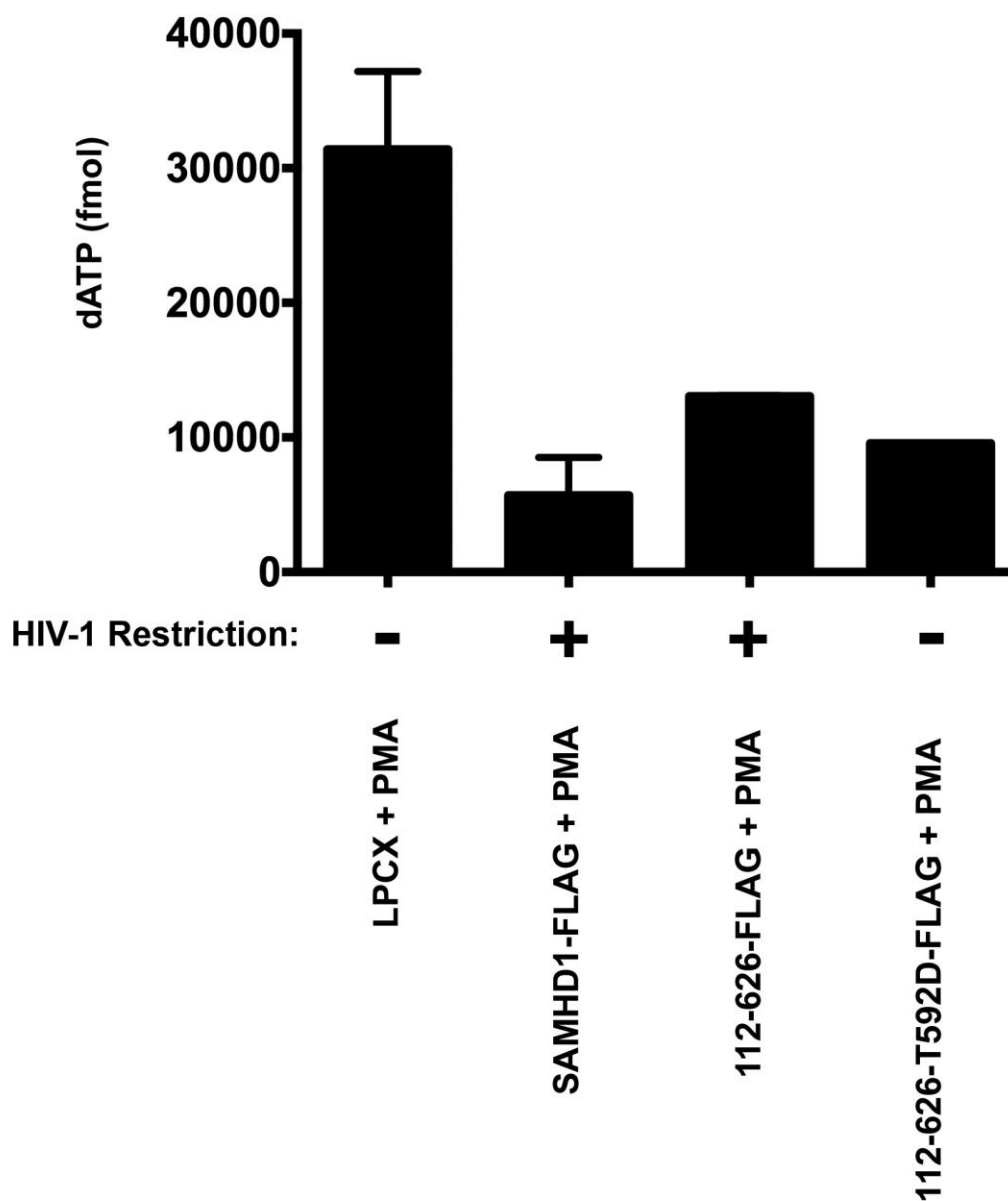




**Figure S2. Characterization of SAMHD1 Variants, Related to Figure 3**

U937 cells stably expressing the indicated SAMHD1 variants **(A)** were challenged with increasing amounts of HIV-1-GFP **(B)**, and infection was determined by measuring the percentage of GFP-positive cells by flow cytometry as described in Experimental Procedures. HeLa cells stably expressing the indicated SAMHD1 variants **(C)** were challenged with increasing amounts of HIV-1-GFP **(D)** to measure infectivity. **(E)** Similarly, U937 cells stably expressing the indicated SAMHD1 variant were challenged with increasing amounts of HIV-1-GFP. **(F)** HeLa cells were cotransfected with plasmids expressing the indicated SAMHD1 variants and HA-tagged Vpx from HIV-2<sub>ROD</sub> (Vpx<sub>ROD</sub>) or the empty vector. Thirty-six hours post-transfection cells were harvested, and the

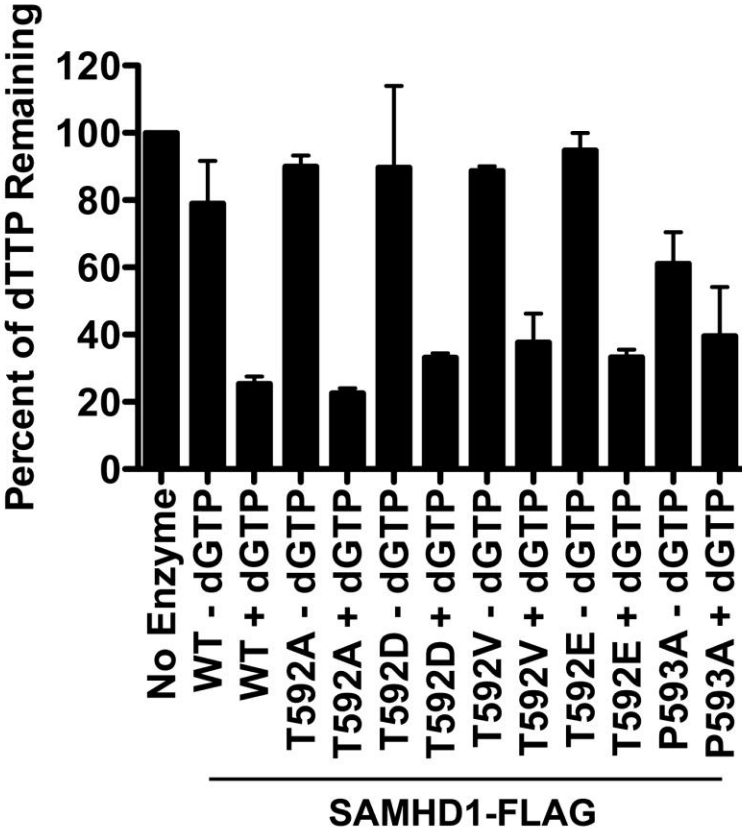
expression levels of SAMHD1 and Vpx were analyzed by Western blot using anti-FLAG and HA antibodies. As a loading control, cell extracts were Western blotted using antibodies against GAPDH. Similar results were obtained in three independent experiments and a representative experiment is shown. **(G)** The oligomerization ability of the different SAMHD1 variants was determined by measuring the interaction between SAMHD1-FLAG and SAMHD1-HA. Human 293T cells were co-transfected with plasmids expressing the indicated SAMHD1-FLAG variant together with the corresponding SAMHD1-HA variant. Cells were lysed 24 hours after transfection and analyzed by Western blotting using anti-HA and anti-FLAG antibodies **(Input)**. Cell lysates were used for immunoprecipitation (IP) with an antibody directed against the FLAG epitope, as described in Experimental Procedures. Elution of the immunocomplexes was performed with a FLAG tripeptide and analyzed by Western blotting (WB) using anti-HA and anti-FLAG antibodies **(immunoprecipitates)**. The results of three independent experiments were similar, and the result of a representative experiment is shown. **(H)** SAMHD1 variants binding to nucleic acids. Human 293T cells were transfected with plasmid expressing the indicated SAMHD1 variant. Lysed cells 24 hours after transfection **(Input)** were incubated with the double-stranded RNA analog ISD-PS immobilized in Strep Tactin Superflow affinity resin. Eluted proteins from the resin were visualized by Western blotting using anti-FLAG antibodies **(Bound)**. Similar results were obtained in three independent experiments and a representative experiment is shown. ISD-PS, interferon-stimulatory DNA sequence containing a phosphorothioate backbone. **(I)** Intracellular distribution of SAMHD1 variants in HeLa cells was measured by fluorescent microscopy. HeLa cells expressing the indicated SAMHD1-FLAG variants were fixed and immunostained using antibodies against FLAG (green) as described in Experimental Procedures. Cellular nuclei were stained by using DAPI (blue). Similar results were obtained in three independent experiments and a representative experiment is shown.



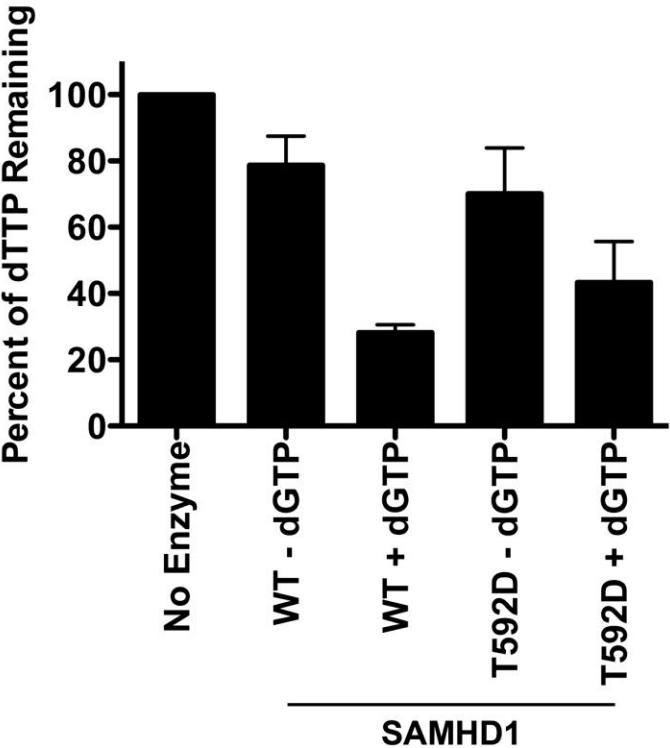
**Figure S3. dTTPase Activity of SAMHD1 Variants, Related to Figure 4**

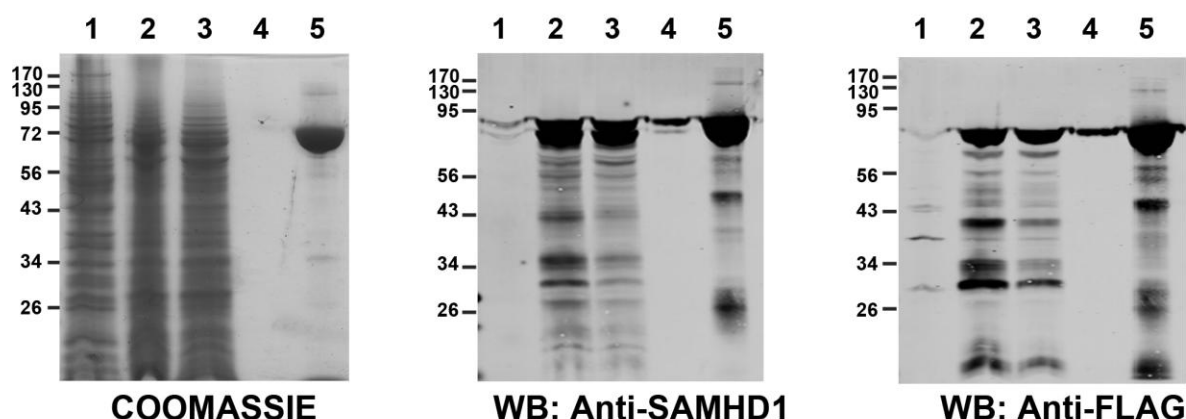
Quantification of dATP levels was performed from PMA-treated U937 cells stably expressing SAMHD1-FLAG, 112-626-FLAG and 112-626-T592D-FLAG, was performed by a primer extension assay, as described in Experimental Procedures. Similar results were obtained in three separate experiments, and standard deviation is shown.

A



B



**C**

**Figure S4. dTTPase Activity of SAMHD1 Phosphorylation Variants and Purification of Recombinant SAMHD1-T592V, Related to Figure 5**

**(A)** Hydrolysis of dTTP by the different SAMHD1 variants was determined measuring the concentrations of dTTP by HPLC in the presence of SAMHD1's allosteric activator dGTP. The concentration of residual dTTP is expressed as a percentage of the initial concentration of dTTP (percentage of dTTP remaining). **(B)** Similarly, hydrolysis of dTTP by recombinant SAMHD1 variants expressed in baculovirus infected cells was measured. The dTTPase activity of the recombinant variants is expressed as the percentage of dTTP remaining. Experiments were repeated three times and standard deviation is shown. **(C)** Purification of recombinant SAMHD1-T592D from insect cells. Coomassie blue-stained SDS-PAGE and Western blot showing the step wise purification of recombinant SAMHD1-T592D protein from baculovirus-infected Sf9 insect cells. Lane 1, soluble lysate from control Sf9 cells; lane 2, soluble lysate from Sf9 cells infected with baculovirus expressing SAMHD1-T592D; lane 3, soluble lysate after filtration (0.45  $\mu$ m); lane 4, final wash before elution; Lane 5, elution of SAMHD1 with 2.5 mM D-desthiobiotin.

## **SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

### **Cell lines and Plasmids**

Human THP-1(ATCC#TIB-202) and U937 (ATCC#CRL-1593) cells were grown in RPMI supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Human HeLa cells (ATCC# CCL-2) were grown on DMEM supplemented with 10% fetal bovine serum and 1% (w/v) penicillin/streptomycin. LPCX-SAMHD1-HA and LPCX-SAMHD1-FLAG plasmids expressing the codon optimized SAMHD1 fused to either HA or FLAG epitope were previously described (Brandariz-Nunez et al., 2012). The plasmids expressing SAMHD1 variants were created using specific primers and pLPCX-SAMHD1-FLAG or HA as template. PCR products were digested and cloned into the EcoRI and ClaI sites of pLPCX. Orientation of the inserts was confirmed by sequencing and restriction analysis.

### **Generation of U937 cells stably expressing SAMHD1 variants**

Retroviral vectors encoding wild type or mutant SAMHD1 proteins fused to FLAG were created using the LPCX vector (Clontech). Recombinant viruses were produced in 293T cells by co-transfecting the LPCX plasmids with the pVPack-GP and pVPack-VSV-G packaging plasmids (Clontech). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells (Yee et al., 1994). Transduced human monocytic U937 cells were selected in 0.4 µg /ml puromycin (Sigma).

### **Protein analysis**

Cellular proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer as

Supplemental Information

previously described (Lienlaf et al., 2011). Detection of proteins by Western blotting was performed using anti-FLAG (Sigma), anti-SAMHD1 (Abnova), anti-GAPDH (Ambion) or anti-HA (Sigma) antibodies. Secondary antibodies against rabbit and mouse conjugated to Alexa Fluor 680 were obtained from Li-Cor. Bands were detected by scanning blots using the Li-Cor Odyssey Imaging System in the 700 nm channel.

### **Infection with retroviruses expressing the green fluorescent protein (GFP)**

Recombinant retroviruses expressing GFP, pseudotyped with the VSV-G glycoprotein, were prepared as described (Diaz-Griffero et al., 2008). For infections,  $6 \times 10^4$  cells were seeded in 24-well plates and treated with 10ng/ml phorbol-12-myristate-3-acetate (PMA) for 16 hours. PMA stock solution was prepared in DMSO at 250  $\mu$ g/ml. Subsequently, cells were incubated with the indicated retrovirus for 48 hours at 37°C. The percentage of GFP-positive cells was determined by flow cytometry (Becton Dickinson). Viral stocks were titrated by serial dilution on dog Cf2Th cells.

### **SAMHD1 oligomerization assay**

Approximately  $1.0 \times 10^7$  human 293T cells were cotransfected with plasmids encoding SAMHD1 variants tagged with FLAG and HA. After 24 hours, cells were lysed in 0.5 ml of whole-cell extract (WCE) buffer (50 mM Tris [pH 8.0], 280 mM NaCl, 0.5% IGEPAL, 10% glycerol, 5mM MgCl<sub>2</sub>, 50  $\mu$ g/ml ethidium bromide, 50 U/ml benzonase [Roche]). Lysates were centrifuged at 14,000 rpm for 1 h at 4°C. Post-spin lysates were then pre-cleared using protein A-agarose (Sigma) for 1 h at 4°C; a small aliquot of each of these lysates was stored as Input. Pre-cleared lysates containing the tagged proteins were incubated with anti-FLAG-agarose beads (Sigma) for 2 h at 4°C. Anti-FLAG-agarose beads were washed three times in WCE buffer, and immune complexes were eluted using 200  $\mu$ g of FLAG tripeptide/ml in WCE buffer. The eluted samples were separated by SDS-PAGE and analyzed by Western blotting using either anti-HA or anti-FLAG antibodies (immunoprecipitates).

## Nucleic-acid binding assay

Nucleic-acid binding assay was performed as previously described (Goncalves et al., 2012; White et al., 2012). In brief, the synthetic DNA phosphorothioate-containing interferon-stimulatory DNA (ISD-PS), which is an RNA analog, was synthesized with a 5'-biotin tag using the following primers: ISD sense 5'-tacagatctactagtgatctatgactgatctgtacatgatctaca-3', and ISD antisense 5'-tgtagatcatgtacagatcagtcacatagatcactagtagatctgta-3'.

Sense and antisense primers were incubated at 65 °C for 20 minutes, and primers were allowed to anneal by cooling down to room temperature. Annealed primers were immobilized on a Ultralink Immobilized Streptavidin Plus Gel (Pierce). Cells were lysed using TAP lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 5% glycerol, 0.2% NP-40, 1.5 mM MgCl<sub>2</sub>, 25 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, protease inhibitors) and lysates were cleared by centrifugation. Cleared lysates (Input) were incubated with immobilized nucleic acids at 4°C on a rotary wheel for 2h in the presence of 10 µg/ml of Calf-thymus DNA (Sigma) as a competitor. Unbound proteins were removed by three consecutive washes in TAP lysis buffer. Bound proteins to nucleic acids (Bound) were eluted by boiling samples in Laemmli sample buffer (63 mM Tris HCl, 10% Glycerol 2% SDS, 0.0025% Bromophenol Blue) and analyzed by Western blotting using anti-FLAG antibodies (Sigma).

## Cellular dNTPs quantification by a primer extension assay.

2-3 x 10<sup>6</sup> cells were collected for each cell type. Cells were washed twice with 1x PBS, pelleted and resuspended in ice cold 65% methanol in Millipore grade water. Cells were vortexed for 2 minutes and incubated at 95°C for 3 minutes. Cells were centrifuged at 14000 rpm for 3 minutes and the supernatant was transferred to a new tube for the complete drying of the methanol by using a speed vac. The dried samples were resuspended in Millipore grade water. An 18-nucleotide primer labeled at the 5' end with α-<sup>32</sup>P (5'-GTCCCTGTTCTGGGCGCCA-3') was annealed at a 1:2 ratio respectively to four different 19-nucleotide templates (5'-NTGGCGCCCGAACAGGGAC-3'), where 'N' represents

nucleotide variation at the 5' end. Reaction condition contains 200 fmoles of template primer, 2  $\mu$ l of 0.5 mM dNTP mix for positive control or dNTP cell extract, 4  $\mu$ l of excess HIV-1 RT, 25 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 100 mM KCl, 5 mM  $MgCl_2$ , and 10  $\mu$ M oligo(dT) to a final volume of 20  $\mu$ L. The reaction was incubated at 37 °C for 5 minutes before being quenched with 10  $\mu$ L of 40 mM EDTA and 99% (vol/vol) formamide at 95°C for 5 minutes. The extended primer products were resolved on a 14% urea-PAGE gel and analyze using a phosphoimager. The extended products were quantified using QuantityOne software to quantify percent volume of saturation. The quantified dNTP content of each sample was accounted for based on its dilution factor, so that each sample volume was adjusted to obtain a signal within the linear range of the assay (Kim et al., 2012; Lahouassa et al., 2012).

### **dNTPs Hydrolysis Reaction.**

SAMHD1 (5.8  $\mu$ M) was incubated with 1 mM dTTP and 100  $\mu$ M dGTP in reaction buffer (50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM  $MgCl_2$ , 0.1% Triton-X100) for 2 h at 37° C. Reactions were terminated by incubation at 75° C for 10 min and diluted 20-fold into 12.5% acetonitrile containing 58  $\mu$ M dCMP as a reference control. HPLC: Dionex DNAPac PA100 column (4x50 mm) was equilibrated with running buffer (25 mM Tris-HCl pH 8, 0.5% acetonitrile) for 10 min, 99  $\mu$ l sample was injected and eluted with a linear gradient of 240 mM  $NH_4Cl$  for 12 min, run at an isocratic gradient with 240 mM  $NH_4Cl$  for 5 min, and column was again equilibrated with running buffer (Beckman Coulter System Gold 126 Solvent Module). Absorbance was measured with a Beckman Coulter System Gold 166 Detector at 254 nm and dTTP abundance was determined by integrating the area under each peak using 32 Karat 8.0 Software. dTTP levels were normalized by the amount of dCMP detected in each diluted sample.

### **Assay to determine dNTPase activity of SAMHD1**

SAMHD1 purified from insect cells or immunoprecipitated from mammalian cells was incubated with or without 100  $\mu$ M dGTP, with or without 6  $\mu$ M dsRNA analogue, 500  $\mu$ M dTTP and 0.25  $\mu$ l  $\alpha^{32}$ P-dTTP (PerkinElmer) in SAMHD1 reaction buffer (50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1% Triton-X 100) in a 17.5  $\mu$ l final volume. Reactions were initiated by addition of SAMHD1, incubated for 1 h at 37° C, and terminated by incubation for 10 min at 70° C. The no enzyme control reaction and the antarctic phosphatase reaction contained both dGTP and dsRNA. The antarctic phosphatase reaction (2  $\mu$ l, New England BioLabs) was used to show the mobility of monophosphates on the plate as a comparison to triphosphate mobility. Reactions were spotted (0.5  $\mu$ l) on a TLC PEI Cellulose F plate (EMD Chemicals) and separated in a 0.8 M LiCl solvent. Product formation was analyzed on a Bio-Rad Personal Molecular Imager.

### **Transfections and Immunofluorescence microscopy**

Transfections of cell monolayers were performed using Lipofectamine Plus reagent (Invitrogen), according to the manufacturer's instructions. Transfections were incubated at 37 °C for 24 h. Indirect immunofluorescence microscopy was performed as previously described (Diaz-Griffero et al., 2002). Transfected monolayers grown on coverslips were washed twice with PBS1X (137 NaCl mM, KCl 2.7 mM, Na<sub>2</sub>HPO<sub>4</sub> • 2 H<sub>2</sub>O 10 mM, KH<sub>2</sub>PO<sub>4</sub> mM) and fixed for 15 min in 3.9 % paraformaldehyde in PBS1X. Fixed cells were washed twice in PBS1X, permeabilize for 4 min in permeabilizing buffer (0.5% Triton X-100 in PBS), and then blocked in PBS1X containing 2% bovine serum albumin(blocking buffer) for 1h at room temperature. Cells were then incubated for 1h at room temperature with primary antibodies diluted in blocking buffer. After three washes with PBS, cells were incubated for 30 min in secondary antibodies and 1  $\mu$ g of DAPI (49, 69-diamidino-2-phenylindole)/ml. Samples were mounted for fluorescence microscopy by using the ProLong Antifade Kit (Molecular Probes, Eugene, OR). Images were obtained with a Zeiss Observer.Z1 microscope using a 63X objective, and deconvolution was performed using the software AxioVision V4.8.1.0 (Carl

Zeiss Imaging Solutions).

### **Expression and purification of SAMHD1 variants from Sf9 insect cells**

$7.5 \times 10^8$  Sf9 insect cells growing in suspension were infected with 5 PFU/cell of the recombinant baculovirus and incubated at 28°C for 72 h. All subsequent steps were carried out at 4°C. Cells were collected by centrifugation at  $500 \times g$  for 8 min, resuspended in 60 ml of lysis buffer (250 mM NaCl, 50 mM Tris [pH 8.0], 1.5% Triton X-100, 1 mM TCEP, and mammalian protease inhibitor cocktail [Sigma]). The lysate was clarified by centrifugation ( $20,000 \times g$ , 40 min), filtered (0.45  $\mu$ m), and incubated in 1.5 ml StrepTactin Superflow affinity resin (Qiagen). The bound protein was washed 3 three-times with 15 ml of buffer (50 mM NaCl, 50 mM Tris [pH 8.0], 1 mM TCEP) and eluted with washing buffer supplemented with 2.5 mM D-desthiobiotin. The eluate was analyzed by SDS-PAGE and Western blotting using anti-FLAG or anti-SAMHD1 antibodies.

### **Immunoprecipitation and Mass Spectrometry**

$2 \times 10^7$  U937 cells stable expressing SAMHD1-FLAG, THP-1 cells or primary monocyte-derived macrophages were lysed in 1 ml of whole-cell extract buffer (50 mM Tris [pH 8.0], 280 mM NaCl, 0.5% IGEPAL, 10% glycerol, 5mM MgCl<sub>2</sub>, 25 $\mu$ g ethidium bromide, 25U benzonase, EDTA-free protease inhibitor cocktail [Roche], 25mM NaF and phosphatase inhibitor cocktails II and III [Sigma]). Lysates were centrifuged at 14,000 rpm for 1 h at 4°C. Post-spin lysates were then pre-cleared using protein A-agarose (Sigma) for 1 h at 4°C. Pre-cleared lysates were incubated with anti-FLAG-agarose beads or protein A-agarose with the anti-SAMHD1 (Abnova) antibody when appropriate for 2 h at 4 °C to precipitate the FLAG-tagged or endogenous proteins, respectively. Beads containing the immunoprecipitate were washed three times in whole-cell extract buffer. Subsequently, immune complexes were eluted in Laemmli sample buffer by heating to 95 °C for 15 min. The eluted samples

Supplemental Information

were separated by SDS-PAGE and stained with SimplyBlue™ SafeStain (Invitrogen). Bands corresponding to the molecular weight of SAMHD1 were excised and sent to the Taplin Mass Spec Facility (Harvard Medical School) for phosphopeptide mapping.

### **Preparation of resting and replicating CD4<sup>+</sup> T-cells.**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) density gradient separation (specific gravity 1.077 g/ml) for 35 minutes at 800g. After overnight incubation, the non-adherent PBMCs were used for further assays. The cell population contained mostly T-lymphocytes with a small contaminating population of B lymphocytes. PBMCs were cultured in complete RPMI-1640 growth medium (Gibco, Rockville, MD) supplemented with 100 U/ml penicillin (Gibco, Rockville, MD), 100 µg/ml streptomycin (Gibco, Rockville, MD), Glutamax (Gibco, Rockville, MD) and 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Atlanta, GA) and subjected next day to a Dynabeads Untouched Human CD4 T cells purification (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. T-lymphocyte activation and proliferation was achieved by stimulation with anti-human CD3/CD28 (1ug/ml) (EbioScience, San Diego, CA). Cells were seeded in triplicate at a concentration of  $2 \times 10^6$  cells per well in 1000 µl of complete RPMI-1640. 72 hours later cells were pelleted and stored at -80C for further Western Blotting assessment.

### ***In vitro* cdk1 complex kinase assay**

Recombinant GST-SAMHD1 or GST was incubated with recombinant cdk1 complex (Sigma) in phosphorylation buffer (35 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.5 mM EGTA, 100 µM ATP, 1 mM dithiothreitol [DTT], 5 µCi of  $\gamma$ -<sup>32</sup>P-ATP) for 30 min at 30°C. The phosphorylation reaction was stopped by addition of sample buffer. Proteins were resolved on 10% polyacrilamide SDS-PAGE. The resultant gel was dried on a Bio-Rad gel dryer and analyzed using a phosphoimager.

## REFERENCES

- Brandariz-Nunez, A., Valle-Casuso, J.C., White, T.E., Laguette, N., Benkirane, M., Brojatsch, J., and Diaz-Griffero, F. (2012). Role of SAMHD1 nuclear localization in restriction of HIV-1 and SIVmac. *Retrovirology* 9, 49.
- Diaz-Griffero, F., Hoschander, S.A., and Brojatsch, J. (2002). Endocytosis is a critical step in entry of subgroup B avian leukosis viruses. *J Virol* 76, 12866-12876.
- Diaz-Griffero, F., Perron, M., McGee-Estrada, K., Hanna, R., Maillard, P.V., Trono, D., and Sodroski, J. (2008). A human TRIM5alpha B30.2/SPRY domain mutant gains the ability to restrict and prematurely uncoat B-tropic murine leukemia virus. *Virology* 378, 233-242.
- Goncalves, A., Karayel, E., Rice, G.I., Bennett, K.L., Crow, Y.J., Superti-Furga, G., and Burckstummer, T. (2012). SAMHD1 is a nucleic-acid binding protein that is mislocalized due to aicardi-goutieres syndrome-associated mutations. *Hum Mutat*.
- Kim, B., Nguyen, L.A., Daddacha, W., and Hollenbaugh, J.A. (2012). Tight Interplay among SAMHD1 Protein Level, Cellular dNTP Levels, and HIV-1 Proviral DNA Synthesis Kinetics in Human Primary Monocyte-derived Macrophages. *J Biol Chem* 287, 21570-21574.
- Lahouassa, H., Daddacha, W., Hofmann, H., Ayinde, D., Logue, E.C., Dragin, L., Bloch, N., Maudet, C., Bertrand, M., Gramberg, T., *et al.* (2012). SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat Immunol* 13, 223-228.
- Lienlaf, M., Hayashi, F., Di Nunzio, F., Tochio, N., Kigawa, T., Yokoyama, S., and Diaz-Griffero, F. (2011). Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5alpha(rh): structure of the RING domain of TRIM5alpha. *J Virol* 85, 8725-8737.
- White, T.E., Brandariz-Nunez, A., Carlos Valle-Casuso, J., Amie, S., Nguyen, L., Kim, B., Brojatsch, J., and Diaz-Griffero, F. (2012). Contribution of SAM and HD domains to retroviral restriction mediated by human SAMHD1. *Virology*.
- Yee, J.K., Friedmann, T., and Burns, J.C. (1994). Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods Cell Biol* 43 Pt A, 99-112.